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(54) MUTATIONS ASSOCIATED WITH IRON DISORDERS

MUTATIONEN IN VERBINDUNG MIT EISENERKRANKUNGEN

MUTATIONS ASSOCIEES A DES ETATS PATHOLOGIQUES LIES A LA PRESENCE DE FER

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Description

Background of the Invention

[0001] Hemochromatosis is the most common progressive (and sometimes fatal) genetic disease in people of European descent. Hemochromatosis is a disease state characterized by an inappropriate increase in intestinal iron absorption. The increase can result in deposition of iron in organs such as the liver, pancreas, heart, and pituitary. Such iron deposition can lead to tissue damage and functional impairment of the organs.

[0002] In some populations, 60-100% of cases are attributable to homozygosity for a missense mutation at C282Y in the Histocompatibility iron (Fe) loading (HFE) gene, a major histocompatibility (MHC) non-classical class I gene located on chromosome 6p. Some patients are compound heterozygotes for C282Y and another mutation at H63D (Bernard et al., 1998, Am. J. Patholog. 153: 1055-61).

Summary of the Invention

[0003] The invention is based on the discovery of novel mutations which are associated with aberrant iron metabolims, absorption, or storage, or in advanced cases, clinical hemochromatosis. Accordingly, the invention features a method of diagnosing an iron disorder, e.g., hemochromatosis or a genetic susceptibility to developing such a disorder, in a mammal by determining the presence of a mutation in exon 2 of an HFE nucleic acid. The mutation is not a C→G missense mutation at position 187 of SEQ ID NO:1 which leads to a H63D substitution. The nucleic acid is an RNA or DNA molecule in a biological sample taken from the mammal, e.g. a human patient, to be tested. The presence of the mutation is indicative of the disorder or a genetic susceptibility to developing it. An iron disorder is characterized by an aberrant serum iron level, ferritin level, or percent saturation of transferrin compared to the level associated with a normal control individual. An iron overload disorder is characterized by abnormally high iron absorption compared to a normal control individual. Clinical hemochromatosis is defined by an elevated fasting transferrin saturation level of greater than 45% saturation.

[0004] For example, the mutation is a missense mutation at nucleotide 314 of SEQ ID NO:1 such as 314C which leads to the expression of mutant HFE gene product with amino acid substitution I105T. The I105T mutation is located in the α1 helix of the HFE protein and participates in a hydrophobic pocket (the "F" pocket). The alpha helix structure of the α1 domain spans residues S80 to N108, inclusive. The I105T mutation is associated with an iron overload disorder.

Table 1: Human HFE cDNA sequence

```
 atgggcccg cgagccaggc
cggcgcttct cctcctgatg cttttgcaga ccgcggtcct gcaggggcgc ttgctgcgtt
cacactctct gcactacctc ttcatgggtg cctcagagca ggaccttggt ctttccttgt
ttgaagcttt gggctacgtg gatgaccagc tgttcgtgtt ctatgatcat gagaagtcgcc
                                                H63D   S65C
gtgtggagcc ccgaactcca tgggtttcca gtagaatttc aagccagatg tggctgcagc
tgagtcagag tctgaaaggg tgggatcaca tgttcactgt tgacttctgg actattatgg
                  G93R                                  I105T
aaaatcacaa ccacagcaag gagtcccaca ccctgcaggt catcctgggc tgtgaaatgc
aagaagacaa cagtaccgag ggctactgga agtacgggta tgatgggcag gaccaccttg
aattctgccc tgacacactg gattggagag cagcagaacc cagggcctgg cccaccaagc
tggagtggga aaggcacaag attcgggcca ggcagaacag ggcctacctg gagagggact
gccctgcaca gctgcagcag ttgctggagc tggggagagg tgttttggac caacaagtgc
ctccttggt gaaggtgaca catcatgtga cctcttcagt gaccactcta cggtgtcggg
ccttgaacta ctacccccag aacatcacca tgaagtggct gaaggataag cagccaatgg
atgccaagga gttcgaacct aaagacgtat tgcccaatgg ggatgggacc taccagggct
ggataacctt ggctgtaccc cctggggaag agcagagata tacgtgccag gtggagcacc
caggcctgga tcagcccctc attgtgatct gggagccctc accgtctggc accctagtca
ttggagtcat cagtggaatt gctgtttttg tcgtcatctt gttcattgga attttgttca
taatattaag gaagaggcag ggttcaagag gagccatggg gcactacgtc ttagctgaac
gtgagtgaca cgcagcctgc agactcactg tgggaaggag acaaaactag agactcaaag
agggagtgca tttatgagct cttcatgttt caggagagag ttgaacctaa acatagaaat
tgcctgacga actccttgat tttagccttc tctgttcatt tcctcaaaaa gatttcccca
tttaggtttc tgagttcctg catgccggtg atccctagct gtgacctctc ccctggaact
gtctctcatg aacctcaagc tgcatctaga ggcttccttc atttcctccg tcacctcaga
gacatacacc tatgtcattt catttcctat ttttggaaga ggactcctta aatttggggg
acttacatga ttcattttaa catctgagaa aagctttgaa ccctgggacg tggctagtca
taaccttacc agatttttac acatgtatct atgcatttc tggacccgtt caacttttcc
tttgaatcct ctctctgtgt tacccagtaa ctcatctgtc accaagcctt ggggattctt
ccatctgatt gtgatgtgag ttgcacagct atgaaggctg tgcactgcac gaatggaaga
ggcacctgtc ccagaaaaag catcatggct atctgtgggt agtatgatgg gtgtttttag
caggtaggag gcaaatatct tgaaaggggt tgtgaagagg tgtttttct aattggcatg
aaggtgtcat acagatttgc aaagtttaat ggtgccttca tttgggatgc tactctagta

ttccagacct gaagaatcac aataattttc tacctggtct ctccttgttc tgataatgaa
aattatgata aggatgataa aagcacttac ttcgtgtccg actcttctga gcacctactt
acatgcatta ctgcatgcac ttcttacaat aattctatga gataggtact attatcccca
tttctttttt aaatgaagaa agtgaagtag gccgggcacg gtggctcgcg cctgtggtcc
cagggtgctg agattgcagg tgtgagccac cctgcccagc cgtcaaaaga gtcttaatat
atatatccag atggcatgtg tttactttat gttactacat gcacttggct gcataaatgt
ggtacaacca ttctgtcttg aagggcaggt gcttcaggat accatataca gctcagaagt
ttcttcttta ggcattaaat tttagcaaag atatctcatc tcttctttta aaccattttc
ttttttgtg gttagaaaag ttatgtagaa aaaagtaaat gtgatttacg ctcattgtag
aaaagctata aaatgaatac aattaaagct gttatttaat tagccagtga aaaactatta
acaacttgtc tattacctgt tagtattatt gttgcattaa aaatgcatat actttaataa
atgtacattg tattgtaaaa aaaaaaa
```

(SEQ ID NO:1; GENBANK® Accession No. U60319)

Table 2: Human HFE gene product

MGPRARPALLLLMLLQTAVLQG

RLLRSHSLHYLFMGASEQDLGLSLFEALGYVDDQLFVFYDHESRRVEPRTPWVSSRISSQ
MWLQLSQSLKGWDHMFTVDFWTIMENHNHSKESHTLQVILGCEMQEDNSTEGYWKYGYDG
QDHLEFCPDTLDWRAAEPRAWPTKLEWERHKIRARQNRAYLERDCPAQLQQLLELGRGVL
DQQVPPLVKVTHHVTSSVTTLRCRALNYYPQNITMKWLKDKQPMDAKEFEPKDVLPNGDG
TYQGWITLAVPPGEEQRYTCQVEHPGLDQPLIVIWEPSPSGTLVIGVISGIAVFVVILFI
GILFIILRKRQGSRGAMGHYVLAERE (SEQ ID NO: 2; GENBANK® Accession No. U60319)

Residues 1-22 = leader sequence; α1 domain underlined;
residues 63, 65, 93, and 105 indicated in bold type)
Other mutations include nucleotide 277 of SEQ ID NO: 1, e.g., 277C which leads to expression of mutant HFE gene product G93R and one at nucleotide 193 of SEQ ID NO: 1, e.g., 193T, which leads to expression of mutant HFE gene product S65C.

[0005] Any biological sample containing an HFE nucleic acid or gene product is suitable for the diagnostic methods described herein. For example, the biological sample to be analyzed is whole blood, cord blood, serum, saliva, buccal tissue, plasma, effusions, ascites, urine, stool, semen, liver tissue, kidney tissue, cervical tissue, cells in amniotic fluid, cerebrospinal fluid, hair or tears. Prenatal testing can be done using methods used in the art, e.g., amniocentesis or chorionic villa sampling. Preferably, the biological sample is one that can be non-invasively obtained, e.g., cells in saliva or from hair follicles.

[0006] The assay is also used to screen individuals prior to donating blood to blood banks and to test organ tissue, e.g., a donor liver, prior to transplantation into a recipient patient. Both donors and recipients are screened.

[0007] In some cases, a nucleic acid is amplified prior to detecting a mutation. The nucleic acid is amplified using a first oligonucleotide primer which is 5' to exon 2 and a second oligonucleotide primer is 3' to exon 2. To detect mutation at nucleotide 314 of SEQ ID NO: 1, a first oligonucleotide primer which is 5' to nucleotide 314 and a second oligonucleotide primer which is 3' to nucleotide 314 is used in a standard amplification procedure such as polymerase chain reaction (PCR). To amplify a nucleic acid containing nucleotide 277 of SEQ ID NO: 1, a first oligonucleotide primer which is 5' to nucleotide 277 and a second oligonucleotide primer which is 3' to nucleotide 277 is used. Similarly, a nucleic acid containing nucleotide 193 of SEQ ID NO:1 is amplified using primers which flank that nucleotide. For example, for nucleotide 277, the first primer has a nucleotide sequence of SEQ ID NO: 3 and said second oligonucleotide primer has a nucleotide sequence of SEQ ID NO: 4, or the first primer has a nucleotide sequence of SEQ ID NO: 15 and said second oligonucleotide primer has a nucleotide sequence of SEQ ID NO: 16. Table 3, below, shows examples of primer pairs for amplification of nucleic acids in exons and introns of the HFE gene.

Table 3

	I. PRIMERS USED FOR AMPLIFICATION	
Target DNA	Forward Primer	Reverse Primer
Exon 2	CCTCCTACTACACATGGTTAAGG	GCTCTGACAACCTCAGGAAGG
	(SEQ ID NO: 3)	(SEQ ID NO: 4)
Exon 3	GGTGGAAATAGGGACCTATTCC	CACTCTGCCACTAGACTATAGG
	(SEQ ID NO: 5)	(SEQ ID NO: 6)
Exon 4	GTTCCAGTCTTCCTGGCAAGG	AAATGCTTCCCATGGATGCCAG
	(SEQ ID NO: 7)	(SEQ ID NO: 8)
RT-PCR	AAAGGATCCACCATGGGCCCGCGAGCCAGG	GTGAGTCTGCAGGCTGCGTG
	(SEQ ID NO: 9)	(SEQ ID NO: 10)
Intron 4	GTTCCAGTCTTCCTGGCAAGG	AAATGCTTCCCATGGATGCCAG
	(SEQ ID NO: 11)	(SEQ ID NO: 12)
Intron 5	GTTCCAGTCTTCCRGGCAAGG	AAATGCTTCCCATGGATGCCAG
	(SEQ ID NO: 13)	(SEQ ID NO: 14)
	II. PRIMERS USED FOR AMPLIFICATION	
Target DNA	Forward Primer	Reverse Primer
Exon 2	GTGTGGAGCCTCAACATCCTG	ACAAGACCTCAGACTTCCAGC
	(SEQ ID NO: 15)	(SEQ ID NO: 16)
Exon 3	GGTGGAAATAGGGACCTATTCC	CACTCTGCCACTAGAGTATAGG

(continued)

	I. PRIMERS USED FOR AMPLIFICATION	
	(SEQ ID NO: 17)	(SEQ ID NO: 18)
Exon 4	GTTCCAGTCTTCCTGGCAAGG	TTACCTCCTCAGGCACTCCTC
	(SEQ ID NO: 19)	(SEQ ID NO: 20)
RT-PCR	AAAGGATCCACCATGGGCCCGCGAGCCAGG	GTGAGTCTGCAGGCTGCGTG
	(SEQ ID NO: 21)	(SEQ ID NO: 22)
Intron 4	TGCCTGAGGAGGTAATTATGG	AAATGCTTCCCATGGATGCCAG
	(SEQ ID NO: 23)	(SEQ ID NO: 24)
Intron 5	TGCCTGAGGAGGTAATTATGG	AAATGCTTCCCATGGATGCCAG
	(SEQ ID NO: 25)	(SEQ ID NO: 26)

[0008] Mutations in introns of the HFE gene have now been associated with iron disorders and/or hemochromatosis. By "exon" is meant a segment of a gene the sequence of which is represented in a mature RNA product, and by "intron" is meant a segment of a gene the sequence of which is not represented in a mature RNA product. An intron is a part of a primary nuclear transcript which is subsequently spliced out to produce a mature RNA product, i.e., a mRNA, which is then transported to the cytoplasm. A method of diagnosing an iron disorder or a genetic susceptibility to developing the disorder is carried out by determining the presence or absence of a mutation in an intron of HFE genomic DNA in a biological sample. The presence of the mutation is indicative of the disorder or a genetic susceptibility to developing the disorder. The presence of a mutation in an intron is a marker for an exon mutation, e.g., a mutation in intron 4, e.g., at nucleotide 6884 of SEQ ID NO:27 is associated with the S65C mutation in exon 2. A mutation in intron 5, e.g., at nucleotide 7055 of SEQ ID NO:27 is associated with hemochromatosis. In some cases, intron mutations may adversely affect proper splicing of exons or may alter regulatory signals. Preferably, the intron 4 mutation is 6884C and the intron 5 mutation is 7055G. To amplify nucleic acid molecule containing nucleotide 6884 or 7055, primers which flank that nucleotide, e.g., those described in Table 3, are used according to standard methods. Nucleic acid-based diagnostic methods may or may not include a step of amplification to increase the number of copies of the nucleic acid to be analyzed. To detect a mutation in intron 4, a patient-derived nucleic acid may be amplified using a first oligonucleotide primer which is 5' to intron 4 and a second oligonucleotide primer which is 3' to intron 4, and to detect a mutation in intron 5, the nucleic acid may be amplified using a first oligonucleotide primer which is 5' to intron 5 and a second oligonucleotide primer which is 3' to intron 5 (see, e.g., Table 3).

[0009] In addition to nucleic acid-based diagnostic methods, the invention includes a method of diagnosing an iron overload disorder or a genetic susceptibility thereto by determining the presence of a mutation in a HFE gene product in a biological sample. For example, the mutation results in a decrease in intramolecular salt bridge formation in the mutant HFE gene product compared to salt bridge formation in a wild type HFE gene product. The mutation which affects salt bridge formation is at or proximal to residue 63 of SEQ ID NO:2, but is not amino acid substitution H63D. Preferably, the mutation is between residues 23-113, inclusive of SEQ ID NO:2 (Table 2), more preferably, it is between residues 90-100, inclusive, of SEQ ID NO:2, more preferably, it is between residues 58-68, inclusive, of SEQ ID NO:2, and most preferably, the mutation is amino acid substitution S65C. Alternatively, the mutation which affects salt bridge formation is a mutation, e.g., an amino acid substitution at residue 95 or proximal to residue 95 of SEQ ID NO:2. Preferably, the mutation is G93R. Such an HFE mutation is detected by immunoassay or any other ligand binding assay such as binding of the HFE gene product to a transferrin receptor. Mutations are also detected by amino acid sequencing, analysis of the structural conformation of the protein, or by altered binding to a carbohydrate or peptide mimetope.

[0010] A mutation indicative of an iron disorder or a genetic susceptibility to developing such a disorder is located in the α1 helix (e.g., which spans residues 80-108, inclusive, of SEQ ID NO:2) of an HFE gene product. The mutation may be an addition, deletion, or substitution of an amino acid in the wild type sequence. For example, the mutant HFE gene product contains the amino acid substitution I105T or G93R or in the loop of the β sheet of the HFE molecule, e.g., mutation S65C

[0011] Isolated nucleic acids encoding a mutated HFE gene products (and nucleic acids with nucleotide sequences complementary to such coding sequences) are also within the invention. Also included are nucleic acids which are at least 12 but less than 100 nucleotides in length. An isolated nucleic acid molecule is a nucleic acid molecule that is separated from the 5' and 3' sequences with which it is immediately contiguous in the naturally occurring genome of an organism. "Isolated" nucleic acid molecules include nucleic acid molecules which are not naturally occurring. For example, an isolated nucleic acid is one that has been amplified in vitro, e.g, by PCR; recombinantly produced; purified, e.g., by

enzyme cleavage and gel separation; or chemically synthesized. For example, the restriction enzyme, Bst4C I (Sib Enzyme Limited, Novosibirsk, Russia), can be used to detect the G93R mutation (point mutation 277C); this enzyme cuts the mutated HFE nucleic acid but not the wild type HFE nucleic acid. Such nucleic acids are used as markers or probes for disease states. For example, a marker is a nucleic acid molecule containing a nucleotide polymorphism, e.g., a point mutation, associated with an iron disorder disease state flanked by wild type HFE sequences. The invention also encompasses nucleic acid molecules that hybridize, preferably under stringent conditions, to a nucleic acid molecule encoding a mutated HFE gene product (or a complementary strand of such a molecule). Preferably the hybridizing nucleic acid molecule is 400 nucleotides, more preferably 200 nucleotides, more preferably 100, more preferably 50, more preferably 25 nucleotides, more preferably 20 nucleotides, and most preferably 10-15 nucleotides, in length. For example, the nucleotide probe to detect a mutation is 13-15 nucleotides long. The nucleic acids are also used to produce recombinant peptides for generating antibodies specific for mutated HFE gene products. In preferred embodiments, an isolated nucleic acid molecule encodes an HFE polypeptide containing amino acid substitution I105T, G93R, or S65C, as well as nucleic acids the sequence of which are complementary to such nucleic acid which encode a mutant or wild type HFE gene product.

[0012] Also within the invention are substantially pure mutant HFE gene products, e.g., an HFE polypeptide containing amino acid substitution I105T, G93R, or S65C. Substantially pure or isolated HFE polypeptides include those that correspond to various functional domains of HFE or fragments thereof, e.g., a fragment of HFE that contains the α1 domain.

[0013] Wild type HFE binds to the transferrin receptor and regulates the affinity of transferrin receptor binding to transferrin. For example, a C282Y mutation in the HFE gene product reduces binding to the transferrin receptor, thus allowing the transferrin receptor to bind to transferrin (which leads to increased iron absorption).

[0014] The polypeptides of the invention encompass amino acid sequences that are substantially identical to the amino acid sequence shown in Table 2 (SEQ ID NO:2). Polypeptides of the invention are recombinantly produced, chemically synthesized, or purified from tissues in which they are naturally expressed according to standard biochemical methods of purification. Biologically active or functional polypeptides are those which possess one or more of the biological functions or activities of wild type HFE, e.g., binding to the transferrin receptor or regulation of binding of transferrin to the transferrin receptor. A functional polypeptide is also considered within the scope of the invention if it serves as an antigen for production of antibodies that specifically bind to an HFE epitope. In many cases, functional polypeptides retain one or more domains present in the naturally-occurring form of HFE.

[0015] The functional polypeptides may contain a primary amino acid sequence that has been altered from those disclosed herein. Preferably, the cysteine residues in exons 3 and 4 remain unchanged. Preferably the modifications consist of conservative amino acid substitutions. The terms "gene product", "protein", and "polypeptide" are used herein to describe any chain of amino acids, regardless of length or post-translational modification (for example, glycosylation or phosphorylation). Thus, the term "HFE polypeptide or gene product" includes full-length, naturally occurring HFE protein, as well a recombinantly or synthetically produced polypeptide that correspond to a full-length naturally occurring HFE or to a particular domain or portion of it.

[0016] The term "purified" as used herein refers to a nucleic acid or peptide that is substantially free of cellular material, viral material, or culture medium when produced by recombinant DNA techniques, or chemical precursors or other chemicals when chemically synthesized. Polypeptides are said to be "substantially pure" when they are within preparations that are at least 60% by weight (dry weight) the compound of interest. Preferably, the preparation is at least 75%, more preferably at least 90%, and most preferably at least 99%, by weight the compound of interest. Purity can be measured by any appropriate standard method, for example, by column chromatography, polyacrylamide gel electrophoresis, or HPLC analysis.

[0017] Diagnostic kits for identifying individuals suffering from or at risk of developing an iron disorder are also within the invention. A kit for detecting a nucleotide polymorphism associated with an iron disorder or a genetic susceptibility thereto contains an isolated nucleic acid which encodes at least a portion of the wild type or mutated HFE gene product, e.g., a portion which spans a mutation diagnostic for an iron disorder or hemochromatosis (or a nucleic acid the sequence of which is complementary to such a coding sequence). A kit for the detection of the presence of a mutation in exon 2 of an HFE nucleic acid contains a first oligonucleotide primer which is 5' to exon 2 and a second oligonucleotide primer is 3' to exon 2, and a kit for an antibody-based diagnostic assay includes an antibody which preferentially binds to an epitope of a mutant HFE gene product, e.g., an HFE polypeptide containing amino acid substitution I105T, G93R, or S65C, compared to its binding to the wild type HFE polypeptide. An increase in binding of the mutant HFE-specific antibody to a patient-derived sample (compared to the level of binding detected in a wild type sample or sample derived from a known normal control individual) indicates the presence of a mutation which is diagnostic of an iron disorder, i.e., that the patient from which the sample was taken has an iron disorder or is at risk of developing one. The kit may also contain an antibody which binds to an epitope of wild type HFE which contains residue 105, 93, or 65. In the latter case, reduced binding of the antibody to a patient-derived HFE gene product (compared to the binding to a wild type HFE gene product or a gene product derived from a normal control individual) indicates the presence of a mutation which is

diagnostic of an iron disorder, i.e., that the patient from which the sample was taken has an iron disorder or is at risk of developing one.

[0018] Individual mutations and combinations of mutations in the HFE gene are associated with varying severity of iron disorders. For example, the C282Y mutation in exon 4 is typically associated with clinical hemochromatosis, whereas other HFE mutations or combinations of mutations in HFE nucleic acids are associated with disorders of varying prognosis. In some cases, hemochromatosis patients have been identified which do not have a C282Y mutation. The I105T and G93R mutations are each alone associated with an increased risk of iron overload (compared to, e.g., the H63D mutation alone), and the presence of both the I105T and H63D mutation is associated with hemochromatosis. Accordingly, the invention includes a method of determining the prognosis for hemochromatosis in a mammal suffering from or at risk of developing said hemochromatosis by (a) detecting the presence or absence of a first mutation in exon 4 in each allele of an HFE nucleic acid, e.g., patient-derived chromosomal DNA, and (b) detecting the presence of a second mutation in exon 2 in each allele of the nucleic acid. The presence of the first mutation in both chromosomes, i.e. an exon 4 homozygote such as a C282Y homozygote, indicates a more negative prognosis compared to the presence of the second mutation in one or both chromosomes, i.e., an exon 2 heterozygote or homozygote. An exon 4 mutation homozygote is also associated with a more negative prognosis compared to the presence of a first mutation (exon 4) in one allele and the presence of the second mutation (exon 2) in one allele, i.e., a compound heterozygote.

[0019] Other features and advantages of the invention will be apparent from the following detailed description, and from the claims.

Brief Description of the Drawings

[0020]

Fig. 1 is a diagram of the family of proband 1 (HFE genotype H63D/I105T). □ = male, ● = female, ∅ = deceased, ■ = hemochromatosis phenotype. Proband 1 is indicated by an arrow. Phenotype and genotype data: age in year saturation; % Ftn = serum ferritin concentration. 1105 separate chromosomes. The sister of the proband (II, 203) has hyperferritinemia.

Fig. 2 is a diagram of the family of proband 2 (HFE genotype C282Y/G93R). Symbols and abbreviations are the same as those described for Fig. 1. Proband 2 is indicated with an arrow. G93R, C282Y, and wt alleles are known to exist only on separate chromosomes. The father and sister of the proband are being treated for hemochromatosis.

Fig. 3 is a diagram of the family of proband 3 (HFE genotype C282Y/565C). Symbols and abbreviations are the same as those described for Fig. 1. Proband 3 is indicated with an arrow. S65C, C282Y, and wt alleles are know to exist only on separate chromosomes. Proband 3 also has porphyria cutanea tarda, and her brother (II, 203) has ankylosing spondylitis.

Detailed Description

[0021] A proband is the first individual in a family identified to be affected by hemochromatosis. Forward and reverse sequencing of HFE exons 2, 3, 4, and 5, and of portions of HFE introns 2, 4, and 5 was carried out on biological samples taken from twenty hemochromatosis probands who lacked C282Y homozygosity, C282Y/H63D compound heterozygosity, or H63D homozygosity. Four probands had novel HFE coding region mutations. Probands 1 and 2 were heterozygous for previously undescribed mutations: exon 2, nt 314T→C (314C; I105T), and exon 2, nt 277G→C (277C; G93R), respectively; these probands were also heterozygous for H63D and C282Y, respectively. Probands 3 and 4 were heterozygous for an HFE mutation in exon 2, nt 193A→T (193T; S65C). Twelve other probands did not have an exon 2 HFE exon mutation; four were heterozygous for H63D. In probands 1, 2, 3, and 4, the amino acid substitutions I105T, G93R, and S65C (respectively) occurred on separate chromosomes from those with the C282Y or H63D mutations. In 176 normal control subjects, two were heterozygous for S65C; I105T and G93R were not detected in controls. Nine probands were heterozygous and two probands were homozygous for a base-pair change at intron 2, nt 4919T/C (SEQ ID NO:27). Heterozygosity for a base-pair change in intron 4 (nt 6884T→C) was detected only in probands 3 and 4, both of whom also had S65C and HLA-A32. The intron 2 mutation is not diagnostic of an iron disorder and appears randomly in the population. One proband was heterozygous for a base-pair change at intron 5 (nt 7055A→G).

[0022] The data described herein indicate that, in addition to the C282Y and H63D HFE mutations, the HFE exon and intron 5 mutations described herein are diagnostic (and prognostic) of iron disorders.

Pathology of iron overload

[0023] Iron plays an essential role in normal growth and development, but in elevated concentrations, iron is a toxic inorganic molecule and is the leading cause of death in children by poisoning. It has been implicated in the pathophysiology

of a number of common diseases, e.g., hepatitis, cancer, heart disease, reperfusion injury, rheumatoid arthritis, diabetes, AIDS, and psychological abnormalities (e.g. depression).

[0024] The incidence of cancer (especially liver cancer) rises dramatically in the course of hemochromatosis. Iron, acting alone or in synergy with other environmental agents, catalyzes free radical formation. These free radicals which mediate tissue damage also cause DNA double strand breaks and oncogene activation. Iron may also play a role in the pathogenesis of rheumatic diseases and in predisposition to heart disease. High levels of iron can also cause diabetes with 2% of diabetics being hemochromatosis patients. High levels of iron may also affect the disease progression of many viral diseases. Individuals infected with such viruses as hepatitis (e.g., hepatitis B or C) or HIV should be tested for HFE mutations because of the impact increased iron stores have on the treatment and prognosis of such diseases.

[0025] Excessive iron stores and iron deposition is also a major contributing factor in the pathology and treatment of non-valvular heart disease. These conditions include dilated cardiomyopathy cased by deposition of iron in myocardial fibers; myocardial injury the product of anthracycline cardiomyopathy and re-perfusion injury. Increased iron stores may also be a contributing factor in myocardial infarction due to atherosclerosis. Some evidence suggests a significant increase in the incidence of reported heart disease in probands (cardiac symptoms-32%, insulin-dependent diabetes-18%, cardiac arrhythmia-17%, clinically significant coronary artery atherosclerosis-9%, and congestive heart failure-7%. Cardiac complications have been detected in 30% of patients. These include EKG abnormalities, congestive heart failure and cardiac arrhythmias, An increased frequency of HFE mutations in individuals with porphyria cutanea tarda indicates that HFE mutations may predipose an individual to developing this syndrome.

[0026] The effect of iron overload is irreparable damage to vital organs and a multiplicity of associated pathologies described above. The multiplicity of clinical symptoms (and associated pathologies) often causes misdiagnosis of hemochromatosis or failure to diagnose hemochromatosis.

[0027] Untreated hemochromatosis is characterized by iron overload of parenchymal cells, which is toxic and the probable cause of various complications including cirrhosis, and liver cancer, arthropathy, hypogonadotropic hypogonadism, marrow aplasia, skin disorders, diabetes mellitus, and cardiomyopathy. There are 1.5 to 2 million active cases in the U.S. of which 40% have progressive liver disease because they have not been properly diagnosed or treated.

[0028] In untreated hemochromatosis, iron is universally deposited in the hepatocytes of the liver. The iron is found primarily in the cytoplasm of hepatocytes, and by electron microscopy in lysosomal vacuoles, and in more severe cases iron has also been reported deposited in mitochondria. Other liver toxins such as alcohol, and hepatitis exacerbate the damage caused by the iron deposition. Patients with hemochromatosis are advised not to drink, because of increased liver damage, or to smoke, as iron deposition can also occur in the lungs.

[0029] Individuals which are homozygous (and to a lesser extent heterozygous) for an HFE mutation are at risk for developing increased levels of blood lead. Thus, it is important to identify heterozygous as well as homozygous patients.

[0030] Identification and detection of mutations in the HFE gene are critical to understanding the general mechanisms of iron disorders and diagnosing iron-related pathologies.

Nucleic acid-based assays for HFE mutations

[0031] A biological sample containing RNA or DNA is obtained from an individual and the nucleic acid extracted. Optionally, the nucleic acid is amplified according to standard procedures such as PCR. A nucleic acid polymorphism, e.g, a single base pair polymorphism, is detected using methods well known in the art of molecular biology. For example, a mutation is detected using a standard sequencing assay, nucleic acid hybridization, e.g, using standard Southern, Northern, or dot blot hybridization assay systems and an HFE-specific oligonucleotide probe, restriction enzyme fragment polymorphism analysis, oligonucleotide ligation assay (OLA; Nikerson et al., 1990, Nucl. Acids Res. 87:8923-8927), primer extension analysis (Nikiforov et al., 1994, Nucl. Acids Res. 22:4167-4175), single strand conformation polymorphism (SSCP) analysis, allele-specific PCR (Rust et al., 1993, Nucl. Acids Res. 6:3623-3629), denaturing gradient gel electrophoresis (DGGE), fluorescent probe melting curve analysis (Bernard et al., 1998, Am. J. Pathol. 153:1055-61), RNA mismatch cleavage assay, capillary hybridization, or TaqMan™ assay (PE Applied Biosystems, Foster City, CA). Nucleic acid hybridization assays are also carried out using a bioelectronic microchip technology known in the art, e.g., that described in Sosnowski et al., 1997, Proc. Natl. Acad. Sci. U.S.A. 94:1119-1123; Cheng et al. 1998, Nature Biotechnology 16:541-546; or Edman et al., 1997, Nucl. Acids Res. 25:4907-4914.

Detection of mutations using antibodies and other HFE ligands

[0032] Anti-HFE antibodies are know in the art, e.g., those described by Feder et al., 1997, J. Biol. Chem. 272: 14025-14028, or are obtained using standard techniques. Such antibodies can be polyclonal or monoclonal. Polyclonal antibodies can be obtained, for example, by the methods described in Ghose et al., Methods in Enzymology, Vol. 93, 326-327, 1983. An HFE polypeptide, or an antigenic fragment thereof, is used as an immunogen to stimulate the production of HFE-reactive polyclonal antibodies in the antisera of animals such as rabbits, goats, sheep, rodents and the

like. HFE antibodies specific for mutated HFE gene products are raised by immunizing animals with a polypeptide spanning the mutation, e.g, a polypeptide which contains the mutations described herein. For example, the entire α1 domain of a mutant HFE gene product is used as an immunogen. Monoclonal antibodies are obtained by the process described by Milstein and Kohler in Nature, 256:495-97, 1975, or as modified by Gerhard, Monoclonal Antibodies, Plenum Press, 1980, pages 370-371. Hybridomas are screened to identify those producing antibodies that are highly specific for an HFE polypeptide containing a mutation characteristic of an iron metabolism abnormality or clinical hemochromatosis. Preferably, the antibody has an affinity of at least about 10^5 liters/mole, preferably at least 10^6 liters/mole, more preferably at least 10^8 liters/mole, and most preferably, an affinity of at least about 10^9 liters/mole.

[0033] Antibodies specific for the wild type HFE can also be used to diagnose hemochromatosis or iron metabolism abnormalities. Such antibodies are also useful research tools to identify novel mutations indicative of iron disorders or hemochromatosis. A reduction in binding to a wild type HFE-specific antibody indicates the presence of a mutation. Antibody binding is detected using known methods. For example, an ELISA assay involves coating a substrate, e.g., a plastic dish, with an antigen, e.g., a patient-derived biological sample containing an HFE gene product. An antibody preparation is then added to the well. Antibodies specific for a mutant HFE gene product bind or fail to bind to a patient-derived sample in the well. Non-binding material is washed away and a marker enzyme e.g., horse radish peroxidase or alkaline phosphatase, coupled to a second antibody directed against the antigen-specific primary antibody is added in excess and the nonadherent material is washed away. An enzyme substrate is added to the well and the enzyme catalyzed conversion is monitored as indicative of presence of the mutation. Antibodies are also labelled with various sizes of colloidal gold particles or latex particles for detection of binding.

[0034] The invention employs not only intact monoclonal or polyclonal antibodies, but also an immunologically-active antibody fragment, for example, a Fab or $(Fab)_2$ fragment; an antibody heavy chain, an antibody light chain; a genetically engineered single-chain Fv molecule (Ladner et al., U.S. Patent No. 4,946,778).

Example 1: Selection and Characterization of Subjects

[0035] All individuals studied were Caucasians, 18 years of age or older, and from central Alabama. Twenty probands were identified that were either heterozygous for C282Y or H63D, or lacked these mutations. Hemochromatosis is typically diagnosed by detecting elevated saturation of transferrin, with elevated serum ferritin levels, combined with liver biopsy. Each proband patient described below was previously diagnosed to have hemochromatosis by the working diagnostic criterion for hemochromatosis of the American College of Pathologists (elevated fasting transferrin saturation of greater than 60% saturation for males and greater than 50% saturation for females) on at least two occasions in the absence of other known causes. Probands were interviewed regarding their general medical history, diet (including estimated iron content and ethanol consumption), medicinal iron use, receipt of blood transfusion, prior significant hemorrhage, blood donation for transfusion and/or therapeutic phlebotomy, and pregnancy and lactation. Each proband was also evaluated for viral hepatitis B and C and other hepatic disorders, excess ethanol intake, and hereditary, and acquired anemia. Iron overload was defined as evidence of systemic iron overload demonstrated by otherwise unexplained elevated serum ferritin concentration (≥300 ng/mL in men, ≥200 ng/mL in women), increased hepatic iron content determined using hepatic biopsy specimens, or iron >4 g mobilized by phlebotomy. Complications of iron overload were evaluated and treated, and therapeutic phlebotomy was performed using standard methods. HFE mutation analysis for C282Y and H63D and human leukocyte antigen (HLA) immunophenotyping or molecular typing were performed using known methods. In some family members, HLA haplotyping had been performed previously for other disease associations, or their HLA type could be deduced from analysis of their kinship and HFE genotyping results. Measurement of serum iron and other clinical laboratory parameters and analysis of hepatic biopsy specimens were performed using routine methods. Control subjects (n=176) who were in apparently good health and were unrelated to the hemochromatosis probands were recruited from the general population. Iron parameters were measured and HLA typing was performed in two control subjects after HFE genotyping revealed that they had the S65C mutation.

Example 2: HFE Gene Analysis

[0036] PCR amplification was used to detect mutations. Genomic DNA was prepared from peripheral blood buffy coat or saliva using the QIAmpBlood Kit (QIAGEN, Valencia, CA) or FTA Paper and FTA purification reagent (Fitzco Inc., Maple Plain, MN), respectively. Fragments were amplified from genomic DNA using eLONGase (Life Technologies, Gaithersburg, MD) or HotStarTaq DNA polymerase (QIAGEN, Valencia, CA). Primers used to amplify each exon are shown in Table 3.

Table 4: Human HFE genomic DNA

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     caaagtttat
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(SEQ ID NO:27; GENBANK® Accession No. Z92910)

[0037] Exon 1 spans nt 1028-1324, inclusive; exon 2 spans nt 4652-4915, inclusive; exon 3 spans nt 5125-5400, inclusive; exon 4 spans nt 6494-6769, inclusive; exon 5 spans nt 6928-7041, inclusive; exon 6 spans nt 7995-9050, inclusive, and exon 7 spans nt 10206-10637, inclusive. Intron 4 spans nt 6770-6927, inclusive, and intron 5 spans nt 7042-7994, inclusive.

[0038] Total RNA for the RT-PCR was prepared from 1.5 mL of whole blood using the RNeasy Blood Kit (QIAGEN, Valencia, CA). Total messenger RNA encoding the HFE gene was transcribed and amplified with the primers shown above using standard methods, e.g., the Superscript ONE-STEP RT- PCR System (Life Technologies, Gaithersburg, MD). The amplified product was directly subcloned into the pCR2.1-TOPO vector and transfected into TOP 1O bacteria (Invitrogen, Carlsbad, CA). Plasmid DNAs isolated from the subcloning were prepared with the UltraClean Mini Prep Kit (Mo Bio, Solana Beach, CA) and sequenced.

[0039] DNA sequencing was performed using the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems, Foster City, CA) and analyzed on an ABI Prism 377.

[0040] To detect mutations in exon 2 of the HFE gene, the genomic DNA of probands and normal control subjects were amplified and subjected to a dot blot hybridization assay. 1.0 μl of each resulting PCR product was then applied to a Magna Graph nylon membrane (MSI, Westboro, MA). The membranes were treated with 0.5 N NaOH/1.5 M NaCl to denature the DNA, neutralized with 0.5 M Tris-HCl (pH 8.0)/1.5 M NaCl, and rinsed with 2 × SSC (1 × SSC = 0.15 M NaCl/0.015 M sodium citrate, pH 7.0). The DNAs were fixed on the membrane by UV irradiation using a Stratalinker 1800 (Stratagene, Inc., La Jolla, CA). The ECL 3'-oligolabelling and detection system (Amersham, Arlington Heights, IL) was used for synthesis of labeled oligonucleotide probes, hybridization, and signal detection. The oligonucleotide sequences used to detect each point mutation were (substituted bases are shown as upper case letters):

Table 5: Oligonucleotide Probes

Point Mutation	Oligonucleotide
G93R mutation	gtctgaaaCggtgggat (SEQ ID NO:28)
I105T mutation	acttctggactaCtatgg (SEQ ID NO:29)
S65C mutation	atcatgagTgtcgccgt (SEQ ID NO:30)

For signal detection, each oligonucleotide was labeled with fluorescein-11-dUTP using terminal deoxynucleotidyl transferase according to the manufacturer's instructions (Amersham Ltd., Arlington Heights, IL). The membranes were prehybridized in 5 × SSC, 0. 1 % Hybridization buffer component, 0.02% SDS, 5% LiquidBlock at 42°C for approximately 2 hours. Labelled oligonucleotide probes were added to individual bags containing the membranes and prehybridization buffer and incubated at 42°C overnight. The blots were washed twice with 5 × SSC, 0.1 % SDS for 5 minutes at room temperature. Stringency washes for hybridization with oligonucleotides having the sequence of SEQ ID NO: 30 or 28 were performed twice in 0. 2 × SSC/0.1% SDS for 15 minutes at 42°C. Membranes probed with an oligonucleotide having the sequence of SEQ ID NO:29 was washed twice under less stringent conditions (0.5 × SSC/0.1% SDS, 15 minutes at 42°C). Detection of a fluorescent signal was performed according to standard methods.

Example 3: Characterization of Probands

[0041] The mean age of the twenty probands was 44 ± 11 years (range 27-62 years); thirteen (65.0%) were men and seven (35.0%) were women. Eleven had iron overload. One had hepatic cirrhosis, two had diabetes mellitus, four had arthropathy, and two had hypogonadotrophic hypogonadism. One proband also had hereditary stomatocytosis, another had beta-thalassemia trait, a third had ethanol intake >60 g daily, and a fourth had porphyria cutanea tarda. No proband

had evidence of excess oral or parenteral iron intake, or of viral hepatitis B or C. At diagnosis of hemochromatosis, evaluation for common HFE mutations revealed that eleven probands were C282Y heterozygotes, five were H63D heterozygotes, and four did not inherit C282Y or H63D.

[0042] The mean age of the initial 176 control subjects was 52 t 15 years (range 18-86 years); 79 (44.9%) were men and 97 (55.1%) were women. There was no significant difference in the mean ages of men and women. Frequencies of HFE genotypes among the control subjects are shown in Table 6. These values are similar to those previously reported from normal persons from the same geographic area.

Table 6. Frequencies of *HFE* Genotypes in Alabama Subjects.

HFE Genotype	Hemochromatosis Probands with "Atypical" HFE Genotypes, % *(n)*	Normal Control Subjects, % *(n)*
wt/wt	15.00 *(3)*	60.23 *106)*
C282Y/wt	45.00 *(9)*	13.06 *(23)*
H63D/wt	20.00 *(4)*	15.34 *(27)*
S65C/wt	5.00 *(1)*	1.14 *(2)*
C282Y/S65C	5.00 *(1)*	0
C282Y/G93R	5.00 *(1)*	0
H63D/1105T	5.00 *(1)*	0
H63D/C282Y	0	6.82 *(12)*
H63D/H63D	0	3.41 *(6)*
Results are expressed as percentage (n). The wild-type (wt) allele was defined as the *HFE* configuration in which the mutations C282Y, H63D, S65C, I105T, or G93R were not detected.		

Example 4: Identification of novel HFE Mutations in Hemochromatosis Probands

[0043] The following novel mutations (missense mutations) were identified in probands 1 and 2: exon 2, nt 314T→C (I105T), and exon 2, nt 277G→C (G93R), respectively (Table 7; Figs. 1 and 2). Probands 3 and 4 had a S65C mutation The S65C mutation has been observed in hemochromatosis patients but has not been deemed to be indicative of a disease state. In contrast, the data presented herein indicate that the S65C mutation is diagnostic of a disease state. This result is surprising in view of earlier observations. Other than C282Y or H63D, no HFE exon mutations were detected in the remaining sixteen of the twenty probands (Table 6). Nine probands were heterozygous for a base-pair change at intron 2, nt 4919T/C (SEQ ID NO:27); two probands were homozygous for this base-pair change. Heterozygosity for a base-pair change in intron 4 (nt 6884T→C) was detected only in probands 3 and 4, both of whom also inherited S65C. One proband was heterozygous for a base-pair change at intron 5, nt 7055A→G.

[0044] Using dot blot methodology, heterozygosity for the S65C mutation was detected in two of 176 normal control subjects (Table 6). The G93R or I105T mutations were not detected in normal control subjects (Tables 6 and 8).

Example 5: Association of Novel HFE Coding Region Mutations to C282Y and H63D and HFE Intron Alleles

[0045] In proband 1, two mutations of exon 2 (H63D and I105T) were detected. After subcloning the genomic fragment, the subclones revealed that these mutations occurred on separate chromosomes; this observation was confirmed by family studies indicating segregation of I105T

Table 7. Phenotypes and Uncommon *HFE* Genotypes in Alabama Subjects*

Subject †	Age (years), Sex	*NFE* Genotype	HLA Type	Transferrin Saturation, %	Serum Ferritin, ng/mL	Hepatocyte Iron Grade	Phlebotomy, Units
Proband 1	52 M	H63D/I105T	A2, 3; B7,7	62	868	2+	20

(continued)

Subject †	Age (years), Sex	*NFE* Genotype	HLA Type	Transferrin Saturation, %	Serum Ferritin, ng/mL	Hepatocyte Iron Grade	Phlebotomy, Units
Proband 2‡	40 M	C282Y/G93R	A2, 3; B7, 62	78	861	4+	34 37
Proband 35	47 F	C282Y/S65C	A2, 32; B8, 44; Bw4, 6; Cw5, 7	90	281	3+	
Proband 4**	81 F	S65C/wt	A2, 32; B14, 62	100	5,135	N.D.	37
Normal Control 1	28 M	S65C/wt	A2, 31; B35, 60	28	141	N.D.	N.D.
Normal Control 2	69 M	S65C/wt	A24, 26; B8, B37; Bw4, 6; Cw6, 5 (or 7)	42	747	2+	N.D.

*Serum transferrin saturation, serum ferritin concentration, and percutaneous hepatic biopsy were performed before therapeutic phlebotomy was initiated. Reference ranges for these parameters are 15 - 45%; 20 - 300 ng/mL (men) and 20 - 200 ng/mL (women); and 0 -1+, respectively. Iron depletion (serum ferritin ≤ 20 ng/mL) was induced by removing the indicated numbers of units of blood. None of these persons had evidence of hepatic cirrhosis, diabetes mellitus, hemochromatosis-associated arthropathy, hypogonadotrophic hypogonadism, other endocrinopathy, or cardiomopathy. N.D. = not done. The mutations indicated are exon 4, nt 845G→A (C282Y); exon 2, nt 187C→G (H63D); exon 2, nt 314T→C (I105T); exon 2, nt 277G→C (G93R); and exon 2, nt 193A→T (S65C). The wild-type (wt) allele was defined as an HFE allele in which the mutations C282Y, H63D, S65C, I105T, or G93R were not detected.
†Countries of origin: Probands 1 and 2, England; Proband 3, Wales, England, and Americas (Cherokee); Proband 4, England and Ireland; Normal Control 1, England; Normal Control 2, The Netherlands.
‡The father and sister of Proband 2 are presently undergoing therapy for hemochromatosis and iron overload, but their clinical and genetic data were unavailable.
§Proband 3 had porphyria cutanea tarda alleviated with therapeutic phlebotomy.
**Proband 4 had hereditary stomatocytosis unaffected by phlebotomy treatments. 37 units of blood were removed by phlebotomy before treatment was discontinued due to stroke apparently unrelated to anemia or iron overload (post-treatment serum ferritin 1,561 ng/mL). Her 59 year-old daughter (who does not have hereditary stomatocytosis) had transferrin saturation 42%, serum ferritin 62 ng/mL, HLA type Al, 32; B14, 15; Bw4, 6; Cw3, 8, and *HFE* genotype S65C/H63D. These data permitted assignment of the S65C mutation in this family to a haplotype carrying HLA-A32; linkage of S65C and HLA-A32 was also observed in the family of Proband 3.

Table 8. Frequencies of *HFE* Alleles in Alabama Subjects.

	wt*	C282Y	H63D	S65C†	I105T	G93R
Hemochromatosis Probands with "Atypical" *HFE* Genotypes (n = 20)	0.500	0.275	0.125	0.050	0.025	0.025
Normal Control Subjects (n = 176)	0.750	0.099	0.145	0.006	‡	‡

The wild-type (wt) allele was defined as an *HFE* allele in which the mutations C282Y, H63D, S65C, I105T, or G93R were not detected.
†S65C was detected in 2 of 22 (0.091) proband chromosomes and in 2 of 266 (0.0075) control chromosomes that did not bear the C282Y, H63D, S65C, I105T, or G93R mutation.
‡Based on this data set, the frequency of the I105T and G93R HFE alleles is estimated to be < 0.0028, respectively.

and H63D (Fig. 1). In proband 2 (HFE genotype C282Y/G93R), RT-PCR analysis (with subsequent subcloning and sequencing) revealed that these HFE mutations occurred on separate chromosomes. Family studies of proband 3 (HFE

genotype C282Y/S65C) indicated that the C282Y and S65C HFE alleles segregated independently, establishing their occurrence on separate chromosomes (Table 7, Fig. 3).

[0046] In proband 1 (HFE genotype H63D/I105T), the I105T mutation was co-inherited with HLA-A3, B7. In probands 3 and 4 and their respective families, S65C was inherited on the same chromosome as HLA-A32, indicating that HLA-A32 is a marker for chromosomes bearing the S65C mutation, and individuals with HLA-A32 have an increased risk for developing hemochromatosis. The G93R mutation is associated with HLA-A2, and individuals with that haplotype have an increased risk for developing hemochromatosis. The I105T mutation is associated with HLA-A3, e.g., HLA-A3, B7, and individuals with that haplotype have an increased risk for developing hemochromatosis. Among twenty probands tested, the nucleotide polymorphism in intron 4 (nt 6884T→C) was detected in probands 3 and 4, both of whom also had S65C. Subjects that tested positive for the S65C mutation all were found to have the intron 4 (6884T→C) mutation, including two probands (3 and 4), their families, and two normal controls.

Example 6: HFE Coding Region Mutations and Clinical Phenotype

[0047] The I105T and G93R mutations were associated with a hemochromatosis clinical phenotype in probands 1 and 2 who also inherited H63D and C282Y, respectively. Proband 3 had clinical evidence of hemochromatosis, iron overload, and porphyria cutanea tarda associated with compound heterozygosity for C282Y and S65C. Proband 4 had severe iron overload associated with heterozygosity for S65C and co-inheritance of hereditary stomatocytosis (Table 7). The sister of proband 1 (HFE genotype I105T/wt) was not completely evaluated for hyperferritinemia (Fig. 1). Otherwise, family members of probands who were heterozygous for novel HFE mutations described herein had little or no evidence of abnormal iron parameters, a hemochromatosis phenotype, or of iron overload (Table 7 and 9; Figs. 1 and 3). Normal Control 1 who had HFE genotype S65C/wt had a

Table 9. Hemochromatiosis (HC) Family study/patent

Subject/Age/Sex	HLA Type	exon 2	exon 4	intron 4	Tf sat**	Ftn**	Diagnosis/ Hepatocyte
				5636bp	%	ng/ml	Iron grade
Proband 1/57M (201)	A2,3;B7,7	H63D/H, 1105T/1	Wt	T	62	868	HC/2+
brother/45M (204)		H63D/H	Wt	T*	31	186	
sister/50F(203)	A3,3:B7,7	1105T	Wt*	T*	37	576	
daughter/31F (301)	A32,68; B7,44	1105T/1	Wt*	T*	31	56	
son/27M(302)	A2,68; B7,44	H63D/H	wt*	T*	33	44	
Proband 2/40M	A2,3;B7,62	G93R/G	C282Y/C	T	78	861	HC/4+
Father		Wt	C282Y/Y*	T*			HC
Sister		G93R/G	C282Y/C*	T*			HC
Proband 3/47 (201)	A2,32; B8,44	S65C/S	C282Y/C	T/C	90	281	HC/3+
brother/45M (202)	A2,32; B44,51	S65C/S	Wt	T/C	33	42	
mother/81F(102)	A2,2;B8,51	Wt	C282Y/C	T*	NT	NT	
sister/33F(204)	A2,7; B27,51	Wt	Wt	T*	NT	NT	
brother/35M (203)	A2,7; B27,51	Wt	Wt*	T*	NT	NT	
sister		Wt	C282Y/C*	T*			
sister		S65C/S	Wt*	T/C*			

(continued)

Subject/Age/Sex	HLA Type	exon 2	exon 4	intron 4	Tf sat**	Ftn**	Diagnosis/ Hepatocyte
Proband 4/81F	A2,32; B14,62	S65C/S	Wt	T/C	100	S135	HC+stomatocytosis
daughter/59*	A1,32; B14,15	H63D/H, S65C/S	Wt*	T/C	42	62	
Control 1/28M	A2,31; B35,60	S65C/S	Wt	T/C	28	141	
Control 2/69M	A24,26; B8,37	S65C/S	Wt	T/C	42	747	2+
*RE cut **normal (15-45%) ***20-300ng/ml(men) 2C-200ng/ml(women)							

normal iron phenotype (Table 7). Normal Control 2, who also had the HFE genotype S65C/wt, had hyperferritinemia and mildly increased stainable hepatocellular iron deposition, but had no symptoms or other objective findings attributable to iron overload (Table 7). These data indicate that S65C heterozygosity is associated with abnormal iron parameters.

Example 7: HLA gene linkage

[0048] In the family of proband 1, the I105T mutation was linked to HLA-A3, B7, markers which are often linked to the C282Y mutation and its ancestral haplotype. HLA-A3, B7 is also significantly more common among C282Y-negative hemochromatosis probands than in normal control subjects tested. S65C was linked to HLA-A32 in probands 3 and 4 (and their respective families). The base-pair change in intron 4 (nt 6884T→C) was detected only in probands who inherited the S65C mutation. These data indicate that an intron 4 mutation (nt 6884→C) is a marker for chromosomes bearing the S65C HFE allele. Three of four probands who inherited mutated HFE exon 2 mutations described herein also inherited the C282Y or H63D mutations on separate chromosomes. In a fourth proband, the co-inheritance of S65C heterozygosity and hereditary stomatocytosis was associated with severe iron overload.

[0049] Altered interactions of transferrin receptor, transferrin, and C282Y and H63D mutant HFE protein contribute to the pathology of hemochromatosis. The S65C, G93R, and I105T mutations are located within the α1 domain: in the α1 helix of the HFE class I-like heavy chain (I105T and G93R), and at the tip of the A chain loop of the β-pleated sheet (S65C). These mutations affect the overall structure of the HFE gene product, and specifically affect the salt bridge between residues H63 and D95. The I105T substitution also inhibits proper folding of the α1 domain of the HFE gene product, and specifically affects the hydrophobicity of the hydrophobic F pocket.

[0050] Other embodiments are within the following claims.

Claims

1. A method of diagnosing an iron disorder or a genetic susceptibility to developing said disorder in a mammal, comprising determining the presence of a mutation in exon 2 of an HFE nucleic acid in a biological sample from said mammal, wherein said mutation is a missense mutation at nucleotide 314 of SEQ ID NO: 1, a mutation at nucleotide 277 of SEQ ID NO: 1, or a mutation at nucleotide 193 of SEQ ID NO: 1, and wherein the presence of said mutation is indicative of said disorder or a genetic susceptibility to developing said disorder.

2. The method of claim 1, wherein said disorder is hemochromatosis.

3. The method of claim 1 or claim 2, wherein said nucleic acid is a DNA molecule.

4. The method of claim 1 or claim 2, wherein said nucleic acid is a RNA molecule.

5. The method of any preceding claim, wherein said mutation is a missense mutation at nucleotide 314 of SEQ ID NO:1.

6. The method of claim 5, wherein said mutation is 314C.

7. The method of claim 6, wherein said mutation results in expression of mutant HFE gene product 1105T.

8. The method of any one of claims 1 to 4, wherein said mutation is at nucleotide 277 of SEQ ID NO:1.

9. The method of claim 8, wherein said mutation is 277C.

10. The method of claim 9, wherein said mutation results in expression of mutant HFE gene product G93R.

11. The method of claims 1 to 4, wherein said mutation is a mutation at nucleotide 193 of SEQ ID NO: 1.

12. The method of claim 11, wherein said mutation is 193T.

13. The method of claim 12, wherein said mutation results in expression of mutant HFE gene product S65C.

14. The method of any preceding claim, wherein said biological sample is selected from the group consisting of whole blood, cord blood, serum, saliva, plasma, effusions, ascites, urine, stool, buccal tissue, liver tissue, kidney tissue, cerebrospinal fluid, skin, hair and tears.

15. The method of claim 14, wherein said biological sample is whole blood.

16. The method of claim 14, wherein said biological sample is saliva.

17. The method of claim 14, wherein said biological sample is hair.

18. The method any preceding claim, wherein said mammal is a human.

19. The method of any preceding claim, further comprising amplifying said nucleic acid using a first oligonucleotide primer which is 5' to exon 2 and a second oligonucleotide primer is 3' to exon 2.

20. The method of claims 1 to 7 and 14 to 19, further comprising amplifying said nucleic acid using a first oligonucleotide primer which is 5' to nucleotide 314 of SEQ ID NO:1 and a second oligonucleotide primer which is 3' to nucleotide 314 of SEQ ID NO:1.

21. The method of claims 1 to 4, 8 to 10 and 14 to 19 , further comprising amplifying said nucleic acid using a first oligonucleotide primer which is 5' to nucleotide 277 of SEQ ID NO:1 1 and a second oligonucleotide primer which is 3' to nucleotide 277 of SEQ ID NO:1.

22. The method of claims 1 to 4, 11 to 13, and 14 to 19, further comprising amplifying said nucleic acid using a first oligonucleotide primer which is 5' to nucleotide 193 of SEQ ID NO:1 and a second oligonucleotide primer which is 3' to nucleotide 193 of SEQ ID NO:1.

23. The method of any one of claims 19 to 22, wherein said first oligonucleotide primer has a nucleotide sequence of SEQ ID NO:3 and said second oligonucleotide primer has a nucleotide sequence of SEQ ID NO:4.

24. The method of any one of claims 19 to 22, wherein said first oligonucleotide primer has a nucleotide sequence of SEQ ID NO: 15 and said second oligonucleotide primer has a nucleotide sequence of SEQ ID NO:16.

25. A method of diagnosing an iron disorder or a genetic susceptibility to developing said disorder in a mammal, comprising determining the presence or absence of a mutation in an intron of HFE genomic DNA in a biological sample from said mammal, wherein said mutation is at nucleotide 6884 of SEQ ID NO:27 or nucleotide 7055 of SEQ ID NO:27, and wherein the presence of said mutation is indicative of said disorder or a genetic susceptibility to developing said disorder.

26. The method of claim 25, wherein said mutation is at nucleotide 6884 of SEQ ID NO:27.

27. The method of claim 26, wherein said mutation is 6884C.

28. The method of claim 25, wherein said mutation is at nucleotide 7055 of SEQ ID NO:27.

29. The method of claim 28, wherein said mutation is 7055G.

30. The method of any one of claims 25 to 27 further comprising amplifying said nucleic acid using a first oligonucleotide primer which is 5' to intron 4 and a second oligonucleotide primer which is 3' to intron 4.

31. The method of any one of claims 25, 28 and 29 further comprising amplifying said nucleic acid using a first oligonucleotide primer which is 5' to intron 5 and a second oligonucleotide primer which is 3' to intron 5.

32. A method of diagnosing an iron disorder or a genetic susceptibility to developing said disorder in a mammal, comprising determining the presence of a mutation in a HFE gene product in a biological sample from said mammal, wherein said mutation results in a decrease in an intramolecular salt bridge formation in said HFE gene product, wherein said mutation is amino acid substitution I105T of SEQ ID NO: 2, G93R of SEQ ID NO:2, or S65C of SEQ ID NO: 2, and wherein the presence of said mutation is indicative of said disorder or a genetic susceptibility to developing said disorder.

33. The method of claim 28, wherein said disorder is hemochromatosis.

34. The method of claim 28, wherein said mutation is detected by immunoassay.

35. The method of any one of claims 28 to 30, wherein said mutation is I105T.

36. The method of any one of claims 28 to 30, wherein said mutation is G93R.

37. The method of any one of claims 32 to 34, wherein said mutation is S65C.

38. An isolated nucleic acid molecule encoding an HFE polypeptide comprising amino acid substitution I105T or the complement thereof.

39. An isolated nucleic acid molecule encoding an HFE polypeptide comprising amino acid substitution G93R or the complement thereof.

40. A kit for detecting a nucleotide polymorphism associated with an iron disorder or a genetic susceptibility to developing said disorder in a mammal comprising the nucleic acid molecule of claims 38 to 39.

41. A kit for the detection of the presence of a mutation in exon 2 of an HFE nucleic acid comprising first and second oligonucleotide primers selected from the group consisting of:

a first oligonucleotide primer comprising a nucleotide sequence of SEQ ID NO:3 and a second oligonucleotide primer comprising a nucleotide sequence of SEQ ID NO:4; and
a first oligonucleotide primer comprising a nucleotide sequence of SEQ ID NO:15 and said second oligonucleotide primer comprising a nucleotide sequence of SEQ ID NO:16.

42. A substantially pure HFE polypeptide comprising amino acid substitution I105T.

43. A substantially pure HFE polypeptide comprising amino acid substitution G93R.

44. A kit for diagnosing an iron disorder or a genetic susceptibility to developing said disorder in a mammal, comprising an antibody which preferentially binds to an epitope of a mutant HFE gene product, wherein said gene product comprises amino acid substitution I105T or G93R.

Patentansprüche

1. Verfahren zur Diagnose einer Eisen-bedingten Störung oder einer genetischen Prädisposition zur Entwicklung dieser Störung in einem Säuger, wobei man das Vorliegen einer Mutation im Exon 2 einer HFE-Nukleinsäure in einer biologischen Probe des Säugers bestimmt, wobei die Mutation eine Missense-Mutation am Nukleotid 314 von SEQ

ID NO:1, eine Mutation am Nukleotid 277 von SEQ ID NO:1, oder eine Mutation am Nukleotid 193 von SEQ ID NO: 1 ist, und wobei das Vorliegen dieser Mutation auf diese Störung oder eine genetische Prädisposition zur Entwicklung dieser Störung hinweist.

2. Verfahren gemäß Anspruch 1, wobei die Störung Hämochromatose ist.

3. Verfahren gemäß Anspruch 1 oder 2, wobei die Nukleinsäure ein DNA-Molekül ist.

4. Verfahren gemäß Anspruch 1 oder 2, wobei die Nukleinsäure ein RNA-Molekül ist.

5. Verfahren gemäß einem der vorhergehenden Ansprüche, wobei die Mutation eine Missense-Mutation am Nukleotid 314 von SEQ ID NO:1 ist.

6. Verfahren gemäß Anspruch 5, wobei die Mutation 314C ist.

7. Verfahren gemäß Anspruch 6, wobei die Mutation zu einer Expression des mutierten HFE-Genprodukts I105T führt.

8. Verfahren gemäß einem der Ansprüche 1 bis 4, wobei die Mutation am Nukleotid 277 von SEQ ID NO: 1 vorliegt.

9. Verfahren gemäß Anspruch 8, wobei die Mutation 277C ist.

10. Verfahren gemäß Anspruch 9, wobei die Mutation zu einer Expression eines mutierten HFE-Genprodukts G93R führt.

11. Verfahren gemäß einem der Ansprüche 1 bis 4, wobei die Mutation eine Mutation am Nukleotid 193 von SEQ ID NO: 1 ist.

12. Verfahren gemäß Anspruch 11, wobei die Mutation 193T ist.

13. Verfahren gemäß Anspruch 12, wobei die Mutation zu einer Expression des mutierten HFE-Genprodukts S65C führt.

14. Verfahren gemäß einem der vorhergehenden Ansprüche, wobei die biologische Probe ausgewählt ist unter Vollblut, Nabelschnurblut, Serum, Speichel, Plasma, Effusionen, Aszites, Urin, Stuhl, Bukkalgewebe, Lebergewebe, Nierengewebe, Zerebrospinalflüssigkeit, Haut, Haar und Tränen.

15. Verfahren gemäß Anspruch 14, wobei die biologische Probe Vollblut ist.

16. Verfahren gemäß Anspruch 14, wobei die biologische Probe Speichel ist.

17. Verfahren gemäß Anspruch 14, wobei die biologische Probe Haar ist.

18. Verfahren gemäß einem der vorhergehenden Ansprüche, wobei der Säuger ein Mensch ist.

19. Verfahren gemäß einem der vorhergehenden Ansprüche, wobei man weiterhin die Nukleinsäure unter Verwendung eines ersten Oligonukleotid-Primers, der 5' vom Exon 2 gelegen ist, und eines zweiten Oligonukleotid-Primers, der 3' vom Exon 2 gelegen ist, amplifiziert.

20. Verfahren gemäß einem der Ansprüche 1 bis 7 und 14 bis 19, wobei man weiterhin die Nukleinsäure unter Verwendung eines ersten Oligonukleotid-Primers, der 5' vom Nukleotid 314 von SEQ ID NO:1 gelegen ist, und eines zweiten Oligonukleotid-Primers, der 3' vom Nukleotid 314 von SEQ ID NO:1 gelegen ist, amplifiziert.

21. Verfahren gemäß einem der Ansprüche 1 bis 4, 8 bis 10 und 14 bis 19, wobei man weiterhin die Nukleinsäure unter Verwendung eines ersten Oligonukleotid-Primers, der 5' vom Nukleotid 277 von SEQ ID NO:1 gelegen ist, und eines zweiten Oligonukleotid-Primers, der 3' vom Nukleotid 277 von SEQ ID NO:1 gelegen ist, amplifiziert.

22. Verfahren gemäß einem der Ansprüche 1 bis 4, 11 bis 13, und 14 bis 19, wobei man weiterhin die Nukleinsäure unter Verwendung eines ersten Oligonukleotid-Primers, der 5' vom Nukleotid 193 von SEQ ID NO:1 gelegen ist, und eines zweiten Oligonukleotid-Primers, der 3' vom Nukleotid 193 von SEQ ID NO:1 gelegen ist, amplifiziert.

23. Verfahren gemäß einem der Ansprüche 19 bis 22, wobei der erste Oligonukleotid-Primer eine Nukleotidsequenz gemäß SEQ ID NO:3 aufweist, und der zweite Oligonukleotid-Primer eine Nukleotidsequenz gemäß SEQ ID NO:4 aufweist.

24. Verfahren gemäß einem der Ansprüche 19 bis 22, wobei der erste Oligonukleotid-Primer eine Nukleotidsequenz gemäß SEQ ID NO:15 aufweist, und der zweite Oligonukleotid-Primer eine Nukleotidsequenz gemäß SEQ ID NO: 16 aufweist.

25. Verfahren zur Diagnose einer Eisen-bedingten Störung oder einer genetischen Prädisposition zur Entwicklung dieser Störung in einem Säuger, wobei man das Vorliegen oder das Fehlen einer Mutation in einem Intron genomischer DNA von HFE in einer biologischen Probe des Säugers bestimmt, wobei die Mutation am Nukleotid 6884 von SEQ ID NO:27 oder am Nukleotid 7055 von SEQ ID NO:27 vorliegt, und wobei das Vorliegen der Mutation auf diese Störung oder eine genetische Prädisposition zur Entwicklung dieser Störung hinweist.

26. Verfahren gemäß Anspruch 25, wobei die Mutation am Nukleotid 6884 von SEQ ID NO:27 vorliegt.

27. Verfahren gemäß Anspruch 26, wobei die Mutation 6884C ist.

28. Verfahren gemäß Anspruch 25, wobei die Mutation am Nukleotid 7055 von SEQ ID NO:27 vorliegt.

29. Verfahren gemäß Anspruch 28, wobei die Mutation 7055G ist.

30. Verfahren gemäß einem der Ansprüche 25 bis 27, wobei man weiterhin die Nukleinsäure unter Verwendung eines ersten Oligonukleotid-Primers, der 5' vom Intron 4 gelegen ist, und eines zweiten Oligonukleotid-Primers, der 3' vom Intron 4 gelegen ist, amplifiziert.

31. Verfahren gemäß einem der Ansprüche 25, 28 und 29, wobei man weiterhin die Nukleinsäure unter Verwendung eines ersten Oligonukleotid-Primers, der 5' vom Intron 5 gelegen ist, und eines zweiten Oligonukleotid-Primers, der 3' vom Intron 5 gelegen ist, amplifiziert.

32. Verfahren zur Diagnose einer Eisen-bedingten Störung oder einer genetischen Prädisposition zur Entwicklung dieser Störung in einem Säuger, wobei man das Vorliegen einer Mutation in einem HFE-Genprodukt in einer biologischen Probe des Säugers bestimmt, wobei die Mutation zu einer Verringerung einer intramolekularen Salzbrücken-Bildung in dem HFE-Genprodukt führt, wobei diese Mutation die Aminosäuresubstitution I105T von SEQ ID NO:2, G93R von SEQ ID NO:2, oder S65C von SEQ ID NO:2 ist, und wobei das Vorliegen dieser Mutation auf diese Störung oder eine genetische Prädisposition zur Entwicklung dieser Störung hinweist.

33. Verfahren gemäß Anspruch 28, wobei die Störung Hämochromatose ist.

34. Verfahren gemäß Anspruch 28, wobei die Störung über einen Immunoassay nachgewiesen wird.

35. Verfahren gemäß einem der Ansprüche 28 bis 30, wobei die Mutation I105T ist.

36. Verfahren gemäß einem der Ansprüche 28 bis 30, wobei die Mutation G93R ist.

37. Verfahren gemäß einem der Ansprüche 32 bis 34, wobei die Mutation S65C ist.

38. Isoliertes Nukleinsäuremolekül, das ein HFE-Polypeptid kodiert, welches die Aminosäuresubstitution I105T umfasst, oder das Komplement davon.

39. Isoliertes Nukleinsäuremolekül, das ein HFE-Polypeptid kodiert, welches die Aminosäuresubstitution G93R umfasst, oder das Komplement davon.

40. Kit zum Nachweis eines Nukleotid-Polymorphismus, der assoziiert ist mit einer Eisen-bedingten Störung oder einer genetischen Prädisposition zur Entwicklung dieser Störung in einem Säuger, umfassend das Nukleinsäuremolekül gemäß den Ansprüchen 38 bis 39.

41. Kit zum Nachweis des Vorliegens einer Mutation im Exon 2 einer HFE-Nukleinsäure, umfassend erste und zweite

Oligonukleotid-Primer, die ausgewählt sind unter:

einem ersten Oligonukleotid-Primer, umfassend eine Nukleotidsequenz gemäß SEQ ID NO:3, und einem zweiten Oligonukleotid-Primer, umfassend eine Nukleotidsequenz gemäß SEQ ID NO:4, und
einem ersten Oligonukleotid-Primer, umfassend eine Nukleotidsequenz gemäß SEQ ID NO:15, und einem zweiten Oligionuklteotid-Primer, umfassend eine Nukleotidsequenz gemäß SEQ ID NO:16.

42. Im Wesentlichen reines HFE-Polypeptid, umfassend die Aminosäuresubstitution I105T.

43. Im Wesentlichen reines HFE-Polypeptid, umfassend die Aminosäuresubstitution G93R.

44. Kit zur Diagnose einer Eisen-bedingten Störung oder einer genetischen Prädisposition zur Entwicklung dieser Störung in einem Säuger, umfassend einen Antikörper, der vorzugsweise an ein Epitop eines mutierten HFE-Genprodukts bindet, wobei das Genprodukt die Aminosäuresubstitution I105T oder G93R umfasst.

Revendications

1. Procédé de diagnostic d'un trouble lié au fer ou d'une prédisposition génétique à développer ledit trouble chez un mammifère, comprenant la détermination de la présence d'une mutation dans l'exon 2 d'un acide nucléique HFE dans un échantillon biologique provenant dudit mammifère, dans lequel ladite mutation est une mutation faux-sens au niveau du nucléotide 314 de la séquence SEQ ID NO: 1, une mutation au niveau du nucléotide 277 de la séquence SEQ ID NO: 1, ou une mutation au niveau du nucléotide 193 de la séquence SEQ ID NO: 1, et dans lequel la présence de ladite mutation est indicative dudit trouble ou d'une prédisposition génétique à développer ledit trouble.

2. Procédé selon la revendication 1, dans lequel ledit trouble est l'hémochromatose.

3. Procédé selon la revendication 1 ou la revendication 2, dans lequel ledit acide nucléique est une molécule d'ADN.

4. Procédé selon la revendication 1 ou la revendication 2, dans lequel ledit acide nucléique est une molécule d'ARN.

5. Procédé selon l'une quelconque des revendications précédentes, dans lequel ladite mutation est une mutation faux-sens au niveau du nucléotide 314 de la séquence SEQ ID NO: 1.

6. Procédé selon la revendication 5, dans lequel ladite mutation est 314C.

7. Procédé selon la revendication 6, dans lequel ladite mutation se traduit par l'expression d'un produit génique HFE mutant I105T.

8. Procédé selon l'une quelconque des revendications 1 à 4, dans lequel ladite mutation se situe au niveau du nucléotide 277 de la séquence SEQ ID NO: 1.

9. Procédé selon la revendication 8, dans lequel ladite mutation est 277C.

10. Procédé selon la revendication 9, dans lequel ladite mutation se traduit par l'expression d'un produit génique HFE mutant G93R.

11. Procédé selon les revendications 1 à 4, dans lequel ladite mutation est une mutation au niveau du nucléotide 193 de la séquence SEQ ID NO: 1.

12. Procédé selon la revendication 11, dans lequel ladite mutation est 193T.

13. Procédé selon la revendication 12, dans lequel ladite mutation se traduit par l'expression d'un produit génique HFE mutant S65C.

14. Procédé selon l'une quelconque des revendications précédentes, dans lequel ledit échantillon biologique est choisi dans le groupe constitué par le sang complet, le sang ombilical, le sérum, la salive, le plasma, les épanchements, l'ascite, l'urine, les matières fécales, les tissus buccaux, les tissus hépatiques, les tissus rénaux, le liquide céphalo-

rachidien, la peau, les cheveux et les larmes.

15. Procédé selon la revendication 14, dans lequel ledit échantillon biologique est du sang complet.

16. Procédé selon la revendication 14, dans lequel ledit échantillon biologique est de la salive.

17. Procédé selon la revendication 14, dans lequel ledit échantillon biologique est des cheveux.

18. Procédé selon l'une quelconque des revendications précédentes, dans lequel ledit mammifère est un être humain.

19. Procédé selon l'une quelconque des revendications précédentes, comprenant en outre l'amplification dudit acide nucléique au moyen d'une première amorce oligonucléotidique qui est en 5' de l'exon 2 et d'une deuxième amorce oligonucléotidique qui est en 3' de l'exon 2.

20. Procédé selon les revendications 1 à 7 et 14 à 19, comprenant en outre l'amplification dudit acide nucléique au moyen d'une première amorce oligonucléotidique qui est en 5' du nucléotide 314 de la séquence SEQ ID NO: 1 et d'une deuxième amorce oligonucléotidique qui est en 3' du nucléotide 314 de la séquence SEQ ID NO: 1.

21. Procédé selon les revendications 1 à 4, 8 à 10 et 14 à 19, comprenant en outre l'amplification dudit acide nucléique au moyen d'une première amorce oligonucléotidique qui est en 5' du nucléotide 277 de la séquence SEQ ID NO: 1 et d'une deuxième amorce oligonucléotidique qui est en 3' du nucléotide 277 de la séquence SEQ ID NO: 1.

22. Procédé selon les revendications 1 à 4, 11 à 13, et 14 à 19, comprenant en outre l'amplification dudit acide nucléique au moyen d'une première amorce oligonucléotidique qui est en 5' du nucléotide 193 de la séquence SEQ ID NO: 1 et d'une deuxième amorce oligonucléotidique qui est en 3' du nucléotide 193 de la séquence SEQ ID NO: 1.

23. Procédé selon l'une quelconque des revendications 19 à 22, dans lequel ladite première amorce oligonucléotidique a une séquence nucléotidique de la séquence SEQ ID NO: 3 et ladite deuxième amorce oligonucléotidique a une séquence nucléotidique de la séquence SEQ ID NO: 4.

24. Procédé selon l'une quelconque des revendications 19 à 22, dans lequel ladite première amorce oligonucléotidique a une séquence nucléotidique de la séquence SEQ ID NO: 15 et ladite deuxième amorce oligonucléotidique a une séquence nucléotidique de la séquence SEQ ID NO: 16.

25. Procédé de diagnostic d'un trouble lié au fer ou d'une prédisposition génétique à développer ledit trouble chez un mammifère, comprenant la détermination de la présence ou de l'absence d'une mutation dans l'intron d'un ADN génomique HFE dans un échantillon biologique provenant dudit mammifère, dans lequel ladite mutation se situe au niveau du nucléotide 6884 de la séquence SEQ ID NO: 27 ou du nucléotide 7055 de la séquence SEQ ID NO: 27, et dans lequel la présence de ladite mutation est indicative dudit trouble ou d'une prédisposition génétique à développer ledit trouble.

26. Procédé selon la revendication 25, dans lequel ladite mutation se situe au niveau du nucléotide 6884 de la séquence SEQ ID NO: 27.

27. Procédé selon la revendication 26, dans lequel ladite mutation est 6884C.

28. Procédé selon la revendication 25, dans lequel ladite mutation se situe au niveau du nucléotide 7055 de la séquence SEQ ID NO: 27.

29. Procédé selon la revendication 28, dans lequel ladite mutation est 7055G.

30. Procédé selon l'une quelconque des revendications 25 à 27, comprenant en outre l'amplification dudit acide nucléique au moyen d'une première amorce oligonucléotidique qui est en 5' de l'intron 4 et d'une deuxième amorce oligonucléotidique qui est en 3' de l'intron 4.

31. Procédé selon l'une quelconque des revendications 25, 28 et 29, comprenant en outre l'amplification dudit acide nucléique au moyen d'une première amorce oligonucléotidique qui est en 5' de l'intron 5 et d'une deuxième amorce oligonucléotidique qui est en 3' de l'intron 5.

32. Procédé de diagnostic d'un trouble lié au fer ou d'une prédisposition génétique à développer ledit trouble chez un mammifère, comprenant la détermination de la présence d'une mutation dans un produit génique HFE dans un échantillon biologique provenant dudit mammifère, dans lequel ladite mutation se traduit par une réduction de la formation des ponts salins intramoléculaires dans ledit produit génique HFE, dans lequel ladite mutation est une substitution d'acide aminé I105T de la séquence SEQ ID NO: 2, G93R de la séquence SEQ ID NO: 2, ou S65C de la séquence SEQ ID NO: 2, et dans lequel la présence de ladite mutation est indicative dudit trouble ou d'une prédisposition génétique à développer ledit trouble.

33. Procédé selon la revendication 28, dans lequel ledit trouble est l'hémochromatose.

34. Procédé selon la revendication 28, dans lequel ladite mutation est détectée par immunodosage.

35. Procédé selon l'une quelconque des revendications 28 à 30, dans lequel ladite mutation est I105T.

36. Procédé selon l'une quelconque des revendications 28 à 30, dans lequel ladite mutation est G93R.

37. Procédé selon l'une quelconque des revendications 32 à 34, dans lequel ladite mutation est S65C.

38. Molécule d'acide nucléique isolée codant pour un polypeptide HFE comprenant une substitution d'acide aminé I105T ou son complément.

39. Molécule d'acide nucléique isolée codant pour un polypeptide HFE comprenant une substitution d'acide aminé G93R ou son complément.

40. Trousse de détection d'un polymorphisme nucléotidique associé à un trouble lié au fer ou à une prédisposition génétique à développer ledit trouble chez un mammifère, comprenant la molécule d'acide nucléique selon les revendications 38 et 39.

41. Trousse de détection de la présence d'une mutation dans l'exon 2 d'un acide nucléique HFE comprenant des première et deuxième amorces oligonucléotidiques choisies dans le groupe constitué par :

une première amorce oligonucléotidique comprenant une séquence nucléotidique de la séquence SEQ ID NO: 3 et une deuxième amorce oligonucléotidique comprenant une séquence nucléotidique de la séquence SEQ ID NO: 4 ; et

une première amorce oligonucléotidique comprenant une séquence nucléotidique de la séquence SEQ ID NO: 15 et une deuxième amorce oligonucléotidique comprenant une séquence nucléotidique de la séquence SEQ ID NO: 16.

42. Polypeptide HFE sensiblement pur comprenant une substitution d'acide aminé I105T.

43. Polypeptide HFE sensiblement pur comprenant une substitution d'acide aminé G93R.

44. Trousse de diagnostic d'un trouble lié au fer ou d'une prédisposition génétique à développer ledit trouble chez un mammifère, comprenant un anticorps qui se lie de préférence à un épitope d'un produit génique HFE mutant, dans laquelle ledit produit génique comprend une substitution d'acide aminé I105T ou G93R.

Fig. 1

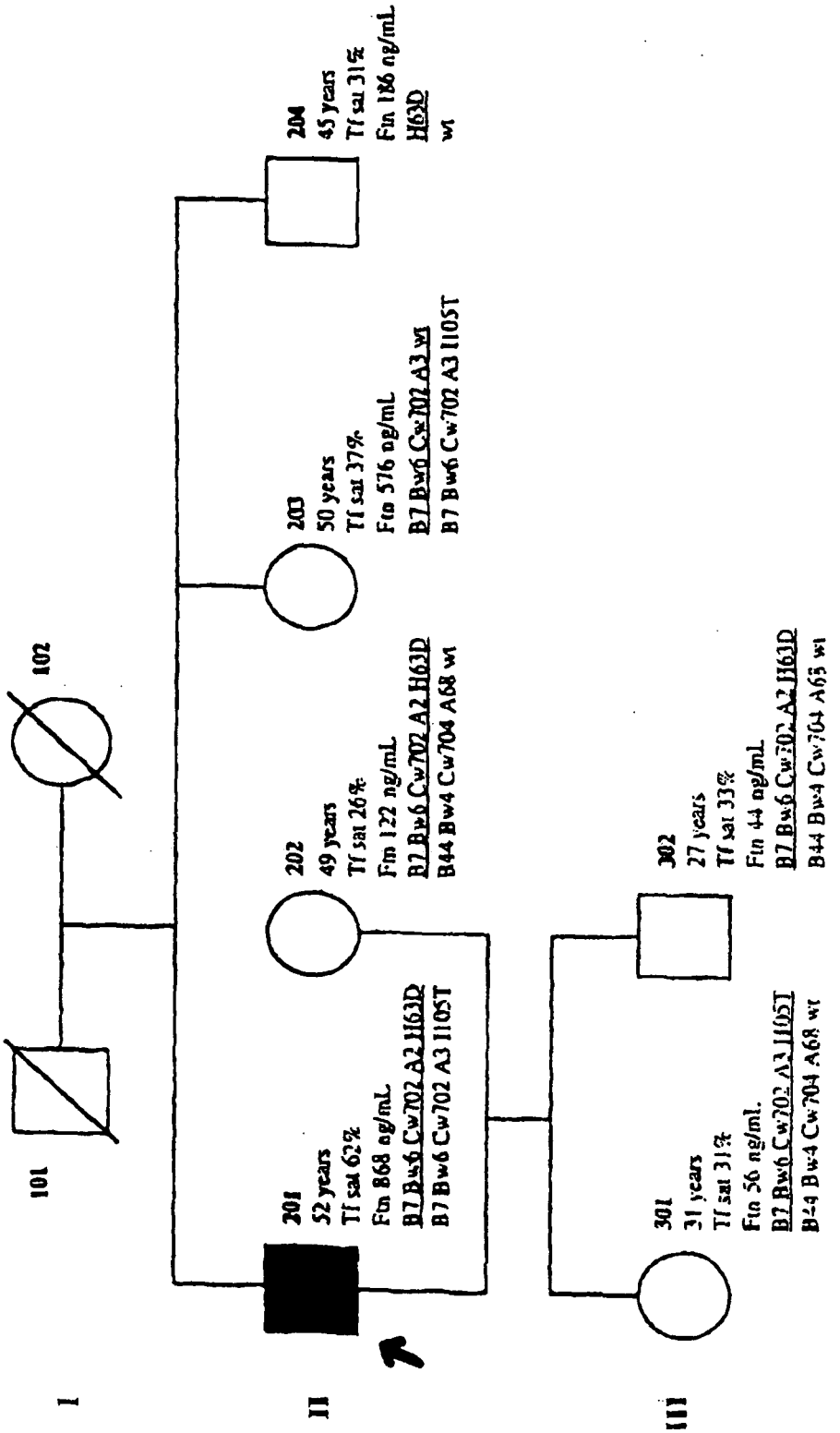

I
II
III
101
102
201
52 years
Tf sat 62%
Fin 868 ng/mL
B7 Bw6 Cw702 A2 H63D
B7 Bw6 Cw702 A3 1105T
202
49 years
Tf sat 26%
Fin 122 ng/mL
B7 Bw6 Cw702 A2 H63D
B44 Bw4 Cw704 A68 wt
203
50 years
Tf sat 37%
Fin 576 ng/mL
B7 Bw6 Cw702 A3 wt
B7 Bw6 Cw702 A3 1105T
204
45 years
Tf sat 31%
Fin 186 ng/mL
H63D
wt
301
31 years
Tf sat 31%
Fin 56 ng/mL
B7 Bw6 Cw702 A3 1105T
B44 Bw4 Cw704 A68 wt
302
27 years
Tf sat 33%
Fin 44 ng/mL
B7 Bw6 Cw702 A2 H63D
B44 Bw4 Cw704 A65 wt

Fig 2

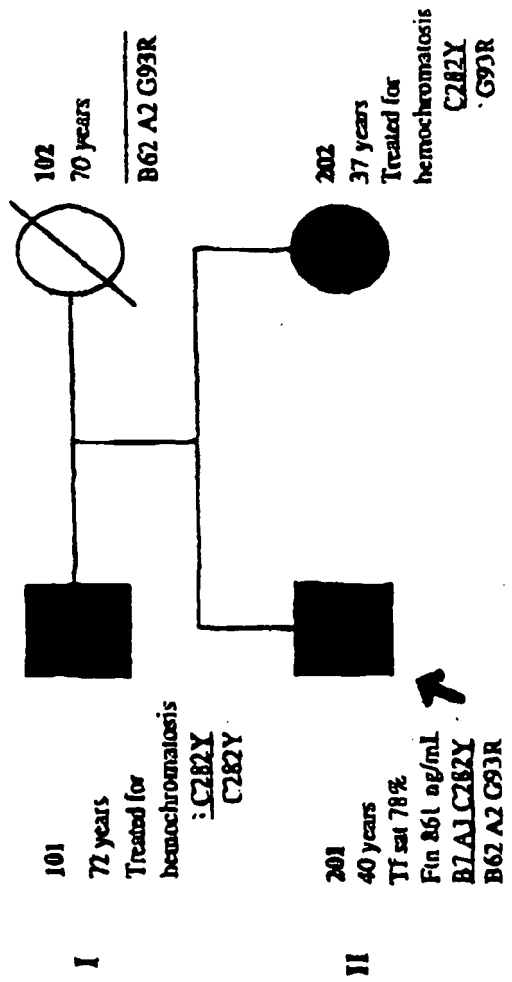
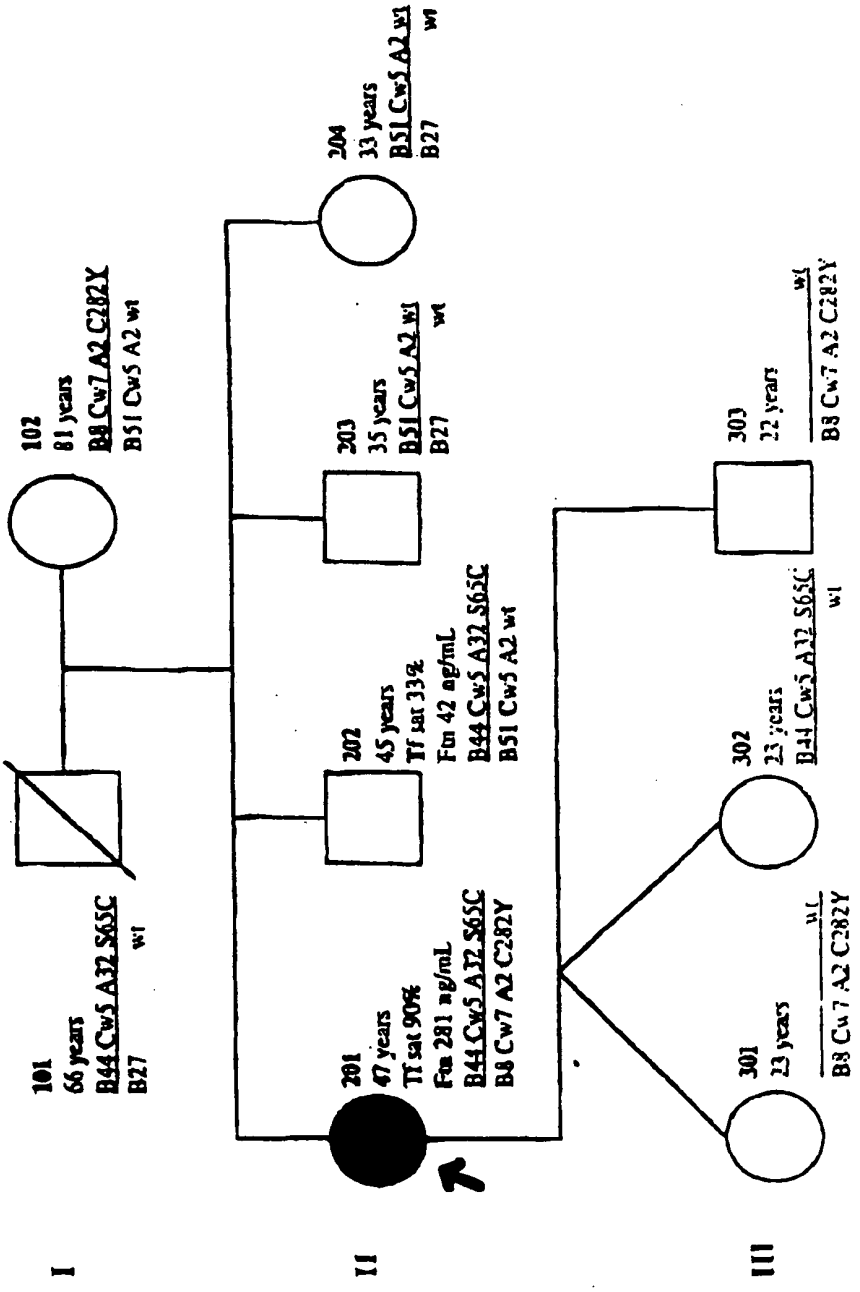

I
II
101
72 years
Treated for
hemochromatosis
: C282Y
C282Y
102
70 years
B62 A2 G93R
201
40 years
Tf sat 78%
Fin 861 ng/mL
B2 A1 C282Y
B62 A2 G93R
202
37 years
Treated for
hemochromatosis
C282Y
G93R

Fig 3


I
II
III
101
66 years
B44 Cw5 A32 S65C
B27
wt
102
81 years
B8 Cw7 A2 C282Y
B51 Cw5 A2 wt
201
47 years
Tf sat 90%
Fer 281 ng/mL
B44 Cw5 A32 S65C
B8 Cw7 A2 C282Y
202
45 years
Tf sat 33%
Fer 42 ng/mL
B44 Cw5 A32 S65C
B51 Cw5 A2 wt
203
35 years
B51 Cw5 A2 wt
B27
wt
204
33 years
B51 Cw5 A2 wt
B27
wt
301
23 years
B8 Cw7 A2 C282Y
wt
302
23 years
B44 Cw5 A32 S65C
wt
303
22 years
B8 Cw7 A2 C282Y
wt

REFERENCES CITED IN THE DESCRIPTION

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